REMARKS

Claims 19, 20, 23, 24, 29-31, 33, 34, 36, 38 and 40-43 are pending and stand ready for further action on the merits. Claims 19, 23, 24, 29-31, 33, 34 and 40 have been withdrawn from consideration as being drawn to non-elected subject matter.

Independent claims 19, 20 and 42 have been amended to recite that the IRES sequence is from a tobamovirus. In addition, claims 44-48 are newly cancelled herein.

No new matter has been added by way of the above amendments.

Interview

Applicants would like to thank Examiner Lambertson for his time and consideration with the telephone interview of September 15, 2004 with Applicants' representatives in the U.S. and Germany. The Applicants found the Examiner's comments very helpful in addressing the issues raised in the Office Action and each of the Examiner's concerns are addressed herein.

Sequence Listing

Applicants' note the Examiner's request that the figures be in a Sequence Listing. An appropriate Sequence Listing is being prepared and will be submitted under separate cover at a later date.

Restriction of the claims

The Examiner maintains the restriction requirement as between:

Group I - Process claims 19, 23, 24, 29-31, 33, 34, 40 and 44; and

Group II - Product claims 20, 36, 38 and 41-43, 45-48.

Applicants respectfully request rejoinder of the withdrawn process claims as being drawn to a process for making a product that is both novel and unobvious. During the interview, the Examiner indicated that if rejoined, the process claims may be subject to rejections under 35 U.S.C.§112, 1st and/or 2nd paragraph.

However Applicants fully believe that the withdrawn process claims are fully compliant with the requirements for patentability under 35 U.S.C.§112, 1st and 2nd paragraphs. Regarding 35 U.S.C.§112, 2nd paragraph, the withdrawn process claims use the same language to describe the claimed features as do the elected claims which have been deemed definite. As such, the elements and features of the process claims are also definite for purposes of 35 U.S.C. §112, 2nd paragraph.

Regarding 35 U.S.C. §112, 1st paragraph, enablement, the specification provides working examples of transgenic plant and animal cell lines made in accordance to the process of independent claim 19. As such, Applicants are claiming no more than they have demonstrated with working examples and the invention is therefore fully enabled. Regarding 35 U.S.C. §112, 1st paragraph, written

description, Applicants are again claiming no more than is described in the specification. As discussed below, the specification fully describes the genus of IRES sequences from tobamoviruses such that one skilled in the art would view the present inventors to be in possession of the claimed genus at the time of the invention. In addition, the examples of the specification fully support transgenic plant and animal cell lines or clones transformed with the IRES sequences of the invention. As such, Applicants sufficiently described how to use the process encompassed by independent claim 19 and the dependent claims thereon so as to fulfill the requirements for written description. Rejoinder and allowance of the withdrawn claims are therefore respectfully requested.

Rejections under 35 U.S.C. §112, 1st paragraph, written description

Claims 20, 36, 38, 41-43 and 45-48 remain rejected under 35 U.S.C.§112, 1st paragraph for lack of written description. In support of the rejection, the Examiner asserts 1) that the specification contains no indication of structural requirements for IRES sequences of the invention, 2) that the specification discloses only a single IRES sequence and 3) that Ivanov et al. teach that the IRES sequence from crTMV is unique even to the genus of tobamoviruses. Applicants traverse this rejection and withdrawal thereof is respectfully requested.

Firstly, Applicants note that the present claims have been amended to be drawn to the genus of IRES sequences from tobamoviruses. The Examiner's assertions in forming the rejection are addressed in turn in relation to the amended claims.

The Examiner asserts that the specification discloses IRES sequences isolated from only a single species of tobamovirus, that being crTMV. The Examiner's assertion in this regard is incorrect because the specification discloses IRES sequences identified by the inventors in three different species of tobamovirus. On a related issue, Applicants note that the conclusion in Ivanov et al. that the IRES from crTMV is unique even to other tobamoviruses is incorrect. Attached hereto is a Declaration of the inventors submitted under 37 C.F.R. §1.132.

With the exception of Timo Korpela all of the present inventors were also authors on the Ivanov et al. article. As such, the inventors have first hand knowledge of the experiments, results and conclusions presented in Ivanov et al. As discussed in the Declaration, due to the experimental conditions used in the experiments of Ivanov et al., the authors/inventors were unable to detect an IRES sequence in TMV U1. In addition, the work in Ivanov et al. preceded the work reported in the present specification and had been submitted with a manuscript in August of 1996, i.e. prior to the Finnish priority date of the present application of May 30, 1997. Subsequent to the submission of the Ivanov et al. manuscript

the inventors determined that certain conclusions that had been reached in Ivanov et al. were, in fact, incorrect.

Specifically, subsequent to the submission of the Ivanov et al. paper, the authors/inventors determined with the experiments that led to the present application, that the failure to detect an IRES sequences in TMV U1 was an experimental artifact and IRES activity was detected for the sequence upstream of the coat protein gene of TMV U1 in yeast cells. It thus turned out that the conclusion of the Ivanov et al. reference that crTMV was unique in having IRES elements was incorrect. Indeed the specification reports the identification of IRES sequences in three different species of tobamovirus.

The genus of tobamovirus is a small one, with roughly 19 species. At the time of the invention approximately ½ of those species had been identified, i.e. less than 10. Thus, the identification of the three IRES sequences in the specification is representative of the claimed genus. That the species disclosed in the specification adequately support the claimed genus is further supported by the common structural elements seen with all the IRES sequences from tobamoviruses.

In this regard, the Examiner asserts that the specification fails to disclose structural requirements for the IRES sequences of the invention. However, a review of the disclosure of the specification reveals that the IRES sequences do, in fact, have

common structural elements. For example, Figures 1B to 1H of the above-captioned application show predicted secondary structures of various tobamoviral IRESs. The 3'-end of the IRESs is defined by the AUG start codon. The RNA sequences forming these IRESs have pronounced self-complementarity, enabling the formation of multiple duplex (base-paired) structures in major parts of the IRES RNA sequences. Major parts of the IRESs are involved in secondary structures. The IRESs form at least one, and typically two to four stem-loops.

As can be further seen from the figures; the stems of the stem-loops can be very large, whereby large stems may be interrupted by bulges. These bulges frequently occur as symmetric bulge loops, i.e. the bulges occur on opposing strand segments and opposing bulges of a bulge loop frequently have the same number of bases. In addition, the primary structures of the IRESs share the common feature of being U-rich.

Coat protein IRESs are typically rich in purine bases in loops that connect stem-loops structures. Such purine-rich loops that connect stem-loop structures typically have more than four bases.

The skilled person envisions that tobamoviral IRESs other than those specifically mentioned and shown is Fig. 1B to 1H would have the same structural properties as described above under item 1 and, as a consequence, will also have IRES activity. As such, the claimed genus of IRES sequences from tobamoviruses for the present

invention is sufficiently described by the recognition of common structural features.

As the concerns raised by the Examiner have been addressed herein with the present amendments and corresponding remarks, withdrawal of the rejection is respectfully requested.

Rejection under 35 U.S.C.§112, 1st paragraph, Enablement

Claims 20, 36, 38, 41-43 and 45-48 remain rejected under 35 U.S.C. §112, 1st paragraph for lack of enablement. The Examiner has taken the position that the specification, while being enabling for an IRES of crTMV origin, does not reasonably provide enablement for any IRES of plant viral origin.

The Examiner maintains the rejection with the assertions that the claimed genus is broader than the enabled scope, that the specification discloses only a single IRES sequence from crTMV and that Ivanov et al. teach that the crTMV IRES sequence is unique even among other tobamoviruses. Applicants traverse this rejection and withdrawal thereof is respectfully requested.

With regard to the scope of the claims, the present invention has been amended to be defined as being IRES sequences from tobamoviruses. This genus is fully enabled by the specification. As discussed above, the specification discloses IRES sequences from three different tobamoviruses and all of the identified IRES sequences share common features, such that one skilled in the art

would conclude that IRES sequences could easily be isolated from other tobamoviruses using the same techniques as those disclosed in the specification. As further discussed above, the report in Ivanov et al. that the crTMV IRES sequence is unique even among tobamoviruses was erroneous and later found by the inventors to be an experimental artifact. As such, the present invention, as claimed, is fully enabled and withdrawal of the rejection is respectfully requested.

Attached hereto is another publication, Skulschev et al., Virology, Vol. 236 (1999) pp. 130-154, which the Examiner may want to make of record before allowing this application.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact MaryAnne Armstrong, PhD, (Reg. No. 40,069) at the telephone number of the undersigned below.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§ 1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

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4360-0102P

Attachment(s): 1) Declaration under 37 C.F.R. §1.132
2) Skulschev et al., Virology, Vol. 236 (1999) pp. 130-154.

Virology 263, 139–154 (1999)
Article ID viro.1999.9928, available online at http://www.idealibrary.com on IDEAL®

Internal Initiation of Translation Directed by the 5′-Untranslated Region of the Tobamovirus Subgenomic RNA I_2

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Previously we reported that, unlike RNA of typical tobamoviruses, the translation of the coat protein (CP) gene of a crucifer-infecting tobamovirus (crTMV) *in vitro* occurred by an internal ribosome entry mechanism mediated by the 148-nt region that contained an internal ribosome entry site ($IRES_{CP,148}^{CR}$). The equivalent 148-nt sequence from TMV U1 RNA ($U1_{CP,148}^{SP}$) was incapable of promoting internal initiation. In the present work, we have found that the 228-nt region upstream of the movement protein (MP) gene of crTMV RNA ($IRES_{MP,228}^{CR}$) contained an IRES element that directed *in vitro* translation of the 3′-proximal reporter genes from chimeric dicistronic transcripts. Surprisingly, the equivalent 228-nt sequence upstream from the MP gene of TMV U1 directed translation of the downstream gene of a dicistronic transcripts as well. Consequently this sequence was termed $IRES_{MP,228}^{U1}$. It was shown that $IRES_{MP,228}^{CR}$, $IRES_{MP,228}^{U1}$, and $IRES_{CP,148}^{CR}$ could mediate expression of the 3′-proximal GUS gene from dicistronic 35S promoter-based constructs *in vivo* in experiments on transfection of tobacco protoplasts and particle bombardment of *Nicotiana benthamiana* leaves. The results indicated that an IRES element was located within the 75-nt region upstream of MP gene ($IRES_{MP,75}$), which corresponded closely to the length of the 5′UTR of TMV subgenomic RNA (sgRNA) I_2. The RNA transcripts structurally equivalent to I_2 sgRNAs of TMV U1 and crTMV, but containing a hairpin structure (H) immediately upstream of $IRES_{MP,75}$ ($HIRES_{MP,75}^{CR}$-MP-CP-3′UTR; $HIRES_{MP,75}^{U1}$-MP-CP-3′UTR), were able to express the MP gene *in vitro*. The capacity of $HIRES_{MP,75}^{CR}$ sequence for mediating internal translation of the 3′-proximal GUS gene *in vivo*, in tobacco protoplasts, was demonstrated. We suggested that expression of the MP gene from I_2 sgRNAs might proceed via internal ribosome entry pathway mediated by $IRES_{MP}$ element contained in the 75-nt 5′UTR. Our results admit that a ribosome scanning mechanism of the MP gene expression from I_2 sgRNA operates concurrently.

Key Words: tobamovirus; MP gene; subgenomic RNA; internal ribosome entry; 5′UTR.

INTRODUCTION

Translation of RNA of tobamoviruses occurs by a scanning mechanism traditional for the majority of eukaryotic mRNAs (for reviews, see Kozak, 1989; Pain, 1996). In accordance with this mechanism, structurally polycistronic tobamovirus RNA is functionally monocistronic, i.e., only the 5′-proximal open reading frame (ORF) encoding the RNA replicative protein and its readthrough product can be translated from genomic RNA (Bruening *et al.*, 1976; Pelham and Jackson, 1976), whereas other genes are expressed from two separate subgenomic RNAs (sgRNAs) (reviewed by Palukaitis and Zaitlin, 1986). The dicistronic intermediate-length RNA-2 called sgRNA I_2 RNA is translated to produce the 30-kDa movement protein (MP) (Bruening *et al.*, 1976; Higgins *et al.*, 1976; Beachy and Zaitlin, 1977; Goelet and Karn, 1982), whereas the 3′-proximal coat protein (CP) gene of I_2 RNA is translationally silent. This gene is expressed only from small monocistronic sgRNA (Beachy and Zaitlin, 1977).

It has been generally accepted that in contrast to genomic RNA, the I_2 RNA of TMV U1 is uncapped (Hunter *et al.*, 1983; Joshi *et al.*, 1983; Lehto *et al.*, 1990). It has been suggested by Lehto *et al.* (1990) that the lack of a 5′ m^7G-cap and the relatively long 5′-untranslated (5′UTR) leader sequence (75 nucleotides, nts) might make the I_2 RNA a poor template for translation compared with the CP sgRNA, which is capped and has a short (9 nts) leader sequence (Guilley *et al.*, 1979).

The genome of a crucifer-infecting tobamovirus (crTMV) contains four traditional genes encoding two components of the replicase (the proteins of 122 and 178 kDa, the readthrough product of 122 kDa), 29-kDa MP and 17-kDa CP (Dorokhov *et al.*, 1993, 1994). It was found that the 148-nt region upstream of the CP gene of crTMV RNA contained an internal ribosome entry site ($IRES_{CP,148}^{CR}$), promoting internal initiation of the CP gene and different reporter genes translation *in vitro*. The equivalent 148-nt sequence from TMV U1 RNA ($U1_{CP,148}^{SP}$) was incapable of mediating an internal *in vitro* translation (Ivanov *et al.*, 1997). The capacity of crTMV $IRES_{CP,148}^{CR}$ for mediating internal translation distinguishes this tobamovirus from the type member of the

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genus, TMV U1. By analogy with crTMV, the 3′-proximal CP gene of potato virus X occurs by a mechanism of internal initiation (Hefferon *et al.*, 1997).

In this study we show that (i) the 228-nt regions upstream of the MP gene of crTMV and TMV U1 RNAs contain an IRES elements that direct expression of the 3′-proximal reporter genes from dicistronic constructs in cell-free translation systems and *in vivo* in tobacco protoplasts; (ii) the 75-nt 5′UTRs of the MP gene of crTMV RNA ($IRES_{MP,75}^{CR}$) and the MP gene of TMV U1 RNA ($IRES_{MP,75}^{U1}$) are able to promote internal initiation of translation of the 3′-proximal reporter genes from the synthetic dicistronic RNA transcripts *in vitro*; and (iii) the RNA transcripts structurally equivalent to I_2 sgRNAs of crTMV and TMV U1 but containing a stable 5′-terminal hairpin immediately upstream of the untranslated $IRES_{MP,75}$ sequence are able to express the MP gene *in vitro*. In addition the capacity of $IRES_{MP,75}^{CR}$ sequence for mediating internal translation of the 3′-proximal GUS gene *in vivo* is demonstrated. It is suggested that translation of the 5′-proximal gene from uncapped dicistronic sgRNA I_2 might proceed by direct binding of ribosomes to nontranslated $IRES_{MP,75}$ sequence upstream from the MP gene.

RESULTS

The usual analysis for IRES activity involves the construction of dicistronic mRNAs in which IRES element is an intergenic region between two reporter genes. In the present work, we applied this test to the second gene in crTMV RNA, the internally located MP gene. It should be noted that the MP gene was translationally silent in full-length genomic crTMV RNA: no 30-kDa MP could be revealed in wheat germ extracts (WGE) or rabbit reticulocyte lysates (RRL) directed by crTMV genomic RNA (data not shown).

Translation of dicistronic CP-MP RNA

The synthetic uncapped T7 RNA transcripts used in these experiments contained the CP gene followed by the downstream MP gene that was separated from the first gene by the 228-nt sequence preceding the AUG codon of the MP gene of crTMV (Figs. 1E and 1F). In other words, the position of CP and MP genes was reversed compared with their location within crTMV genome. Some of the transcripts of this series contained the 51-nt nontranslated leader sequence upstream of the CP gene (Figs. 1A, 1C, and 1E), whereas the other transcripts (Figs. 1B, 1D, and 1F) contained the 5′-terminal polylinker-derived 102-nt sequence upstream from the CP gene. This sequence was predicted to produce a potentially stable hairpin-loop structure (H) upstream of the CP gene (Fig. 1G). The transcripts listed in Fig. 1 were translated in RRL (Fig. 2) and WGE (data not shown). Figure 2 shows that the H sequence abolished translation of the CP gene within monocistronic (HCP) and dicistronic (HCPMP; $HCPIRES_{MP,228}^{CR}MP$) transcripts. Neither of the two cistrons could be translated from dicistronic transcripts HCPMP in which the ORFs were separated by a short polylinker-derived nonphysiological intercistronic spacer and the 5′ terminus was blocked by the H sequence (Figs. 1D and 2). As might be expected, only the first CP gene was translated from dicistronic transcript CPMP analogous to HCPMP but lacking the H hairpin (Figs. 1C and 2). On the other hand, when the 228-nt region preceding the AUG codon of the MP gene of crTMV ($IRES_{MP,228}^{CR}$ in Fig. 1E) was inserted as the intercistronic spacer in the dicistronic transcripts $CPIRES_{MP,228}^{CR}MP$ (Fig. 1E), they were translated as functionally dicistronic message (Fig. 2). It should be noted that the downstream MP gene was efficiently translated from dicistronic $HCPIRES_{MP,228}^{CR}MP$ transcripts (Fig. 2) although the translation of the first CP gene was blocked by H sequence (Fig. 1F). This implies that expression of the 3′-proximal MP gene from synthetic dicistronic transcript is mediated by the $IRES_{MP,228}^{CR}$ element located within the 228-nt sequence upstream of the MP gene and is not due to leaky scanning or termination-reinitiation mechanisms.

Translation of dicistronic H-CP-GUS and H-GFP-obelin chimeric transcripts with different intercistronic sequences

The second type of uncapped dicistronic chimeric constructs contained the 5′-proximal CP gene of crTMV and the 3′-proximal GUS gene. Different sequences summarized in Fig. 3 were inserted between the two coding regions in these constructs. $IRES_{CP,148}^{CR}$ and $IRES_{MP,228}^{CR}$ from crTMV RNA, the 148-nt region $U1_{CP,148}^{SP}$ from TMV U1 RNA (taken as the negative control), and the 228-nt region ($U1_{MP,228}^{SP}$) preceding the AUG codon of the MP gene in TMV U1 RNA. The remaining control monocistronic transcript (Fig. 3E) contained the $IRES_{MP,228}^{CR}$ region as the 5′-leader sequence upstream of the GUS gene. It was found that expression of the 5′-proximal CP gene was completely blocked by the H-structure (Figs. 2 and 4), whereas the second GUS gene was translated from dicistronic $HCPIRES_{MP,228}^{CR}GUS$ transcripts (Figs. 4A and 4B). These results taken together with our previous data (Ivanov *et al.*, 1997) show that the 228-nt sequence upstream of the MP gene and the 148-nt region upstream of the CP gene in crTMV RNA are capable of mediating internal initiation of translation in RRL and WGE.

In a series of experiments, we compared the efficiencies of crTMV $IRES_{MP,228}^{CR}$ and $IRES_{CP,148}^{CR}$ to direct internal initiation of translation under standard conditions. A single dicistronic mRNA was used, the only variable element of which was $IRES_{MP,228}^{CR}$ or $IRES_{CP,148}^{CR}$ inserted into the intercistronic region. Under similar conditions, the $IRES_{MP,228}^{CR}$ was somewhat more efficient in directing

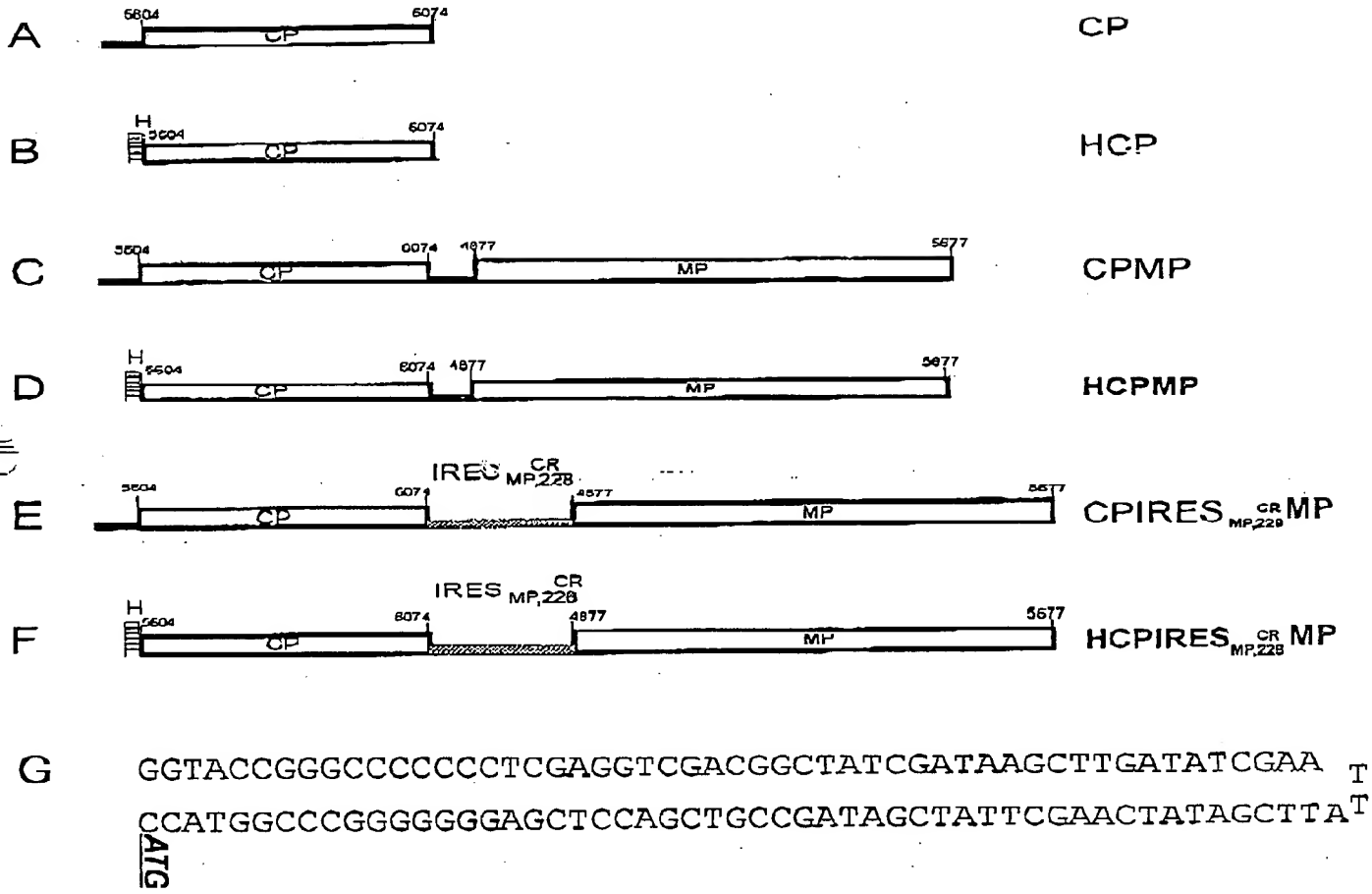

FIG. 1. Schematic representation of the dicistronic CPMP crTMV RNA transcript and its derivatives. (A) CP transcript contains the 5′ leader (51-nt) with no AUG codons upstream from CP gene. (B) HCP, the CP gene with the 102-nt upstream sequence forming a potentially stable hairpin (H). (C) CPMP, dicistronic transcript with 52-nt polylinker-derived nucleotides inserted between the CP and MP genes (construct C) with the 5′-terminal hairpin H. (E) CPIRES$_{MP,228}^{CR}$-MP, the 228-nt sequence located upstream of the MP gene of crTMV was inserted between the CP and MP genes in construct C. (F) HCPIRES$_{MP,228}^{CR}$-MP, dicistronic transcript corresponding to that in E but carrying hairpin H at the 5′ termini. (G) predicted secondary structure of the 5′-terminal stem-loop H. Boxes represent the ORFs, which are drawn in scale. Numbers indicate the corresponding nucleotides of crTMV RNA sequence (Dorokhov *et al.*, 1994).

the expression of GUS gene (Figs. 4A and 4B); however, the results varied significantly in different experiments. To characterize precisely the relative efficiencies of IRES$_{CP}^{CR}$ and IRES$_{MP}^{CR}$ in directing internal translation, the dicistronic transcripts HCPIRES$_{MP,228}^{CR}$GUS and HCPIRES$_{CP,148}^{CR}$GUS were translated in WGE and the enzymatic activities of GUS produced *in vitro* were determined. Figure 5A shows that abilities of IRES$_{MP,228}^{CR}$ and IRES$_{CP,148}^{CR}$ to promote internal translation do not differ substantially.

The transcripts HCPU1$_{MP,228}^{SP}$GUS and HCPIRES$_{MP,228}^{CR}$GUS were compared hoping to demonstrate a functional difference between the equivalent regions located upstream of MP genes in TMV U1 and crTMV RNA by analogy with differences revealed between the U1$_{CP,148}^{SP}$ and IRES$_{CP,140}^{CR}$ mentioned above. It was expected that U1$_{MP,228}^{SP}$ would serve as the equivalent negative control for the IRES$_{MP,228}^{CR}$ sequence in dicistronic constructs. Surprisingly, a significant amount of GUS was produced upon translation of dicistronic constructs HCPU1$_{MP,228}^{SP}$GUS. The level of GUS product accumulation varied in different *in vitro* translation experiments; however, the U1$_{MP,228}^{SP}$ sequence invariably promoted the GUS gene expression despite its 3′-proximal localization (Fig. 4B). These results implied strongly that the 228-nt U1$_{MP,228}^{SP}$ sequence upstream of the MP gene of TMV U1 RNA contained an IRES element. Therefore it was referred to below as IRES$_{MP,228}^{U1}$. In other words, both of

IRES$_{MP,228}^{CR}$ also directed internal translation from dicistronic transcripts (Fig. 6a), however, the efficiency

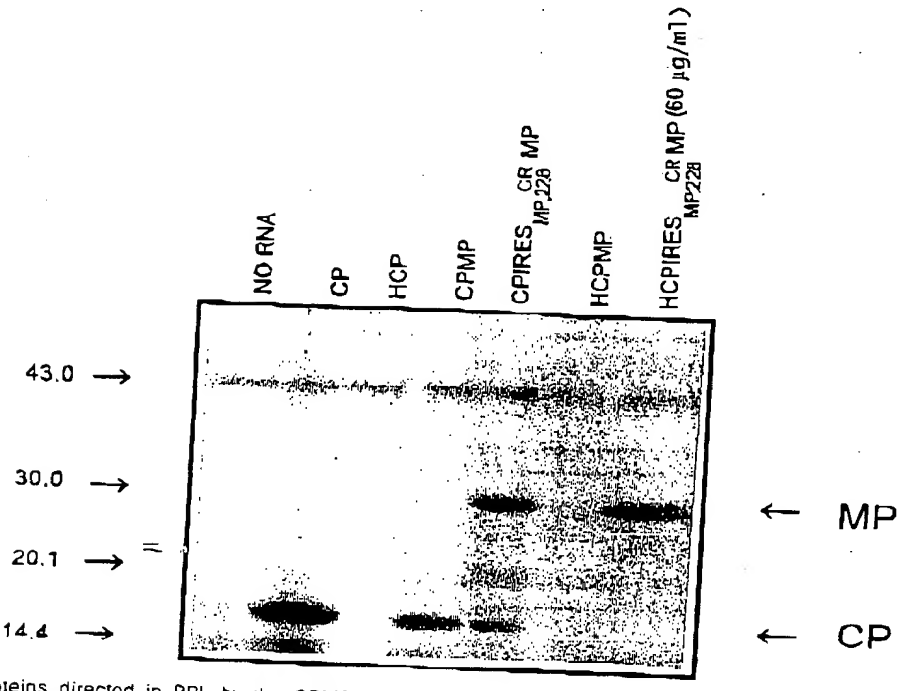

FIG. 2. Analysis of proteins directed in RRL by the CPMP crTMV transcripts. The designations above the panels are described in Fig. 1. Autoradiogram of gradient 8–20% polyacrylamide–SDS gels containing ^{35}S-methionine-labeled products directed by uncapped transcripts in RRL. Concentration of transcripts is 40 µg/ml unless otherwise indicated. The positions of CP and MP are marked and the positions of marker proteins (in kDa) are indicated.

crTMV and TMV U1 contain $IRES_{MP228}$ sequences that are able to mediate internal translation from dicistronic transcripts but are unable to promote the translation of MP gene from full-length genomic RNAs. These conclusions were supported by experiments with the third type of chimeric dicistronic transcripts that contained two non-viral genes: the 5′-proximal GFP gene and the 3′-proximal gene of obelin (jellyfish blue fluorescent protein). The efficiency of different crTMV RNA and TMV U1 RNA sequences to promote internal translation of the 3′-proximal obelin gene was compared quantitatively by determining the obelin activity after translation of dicistronic transcripts in WGE. The data presented in Fig. 5B indicate that the efficiency of the 3′-proximal obelin gene internal translation was similar when mediated by $IRES_{MP228}^{CR}$ (Fig. 5B, column a) and $IRES_{CP146}^{CR}$ (Fig. 5B, column b) intercistronic sequences. It should be emphasized that the efficiency of the equivalent 228-nt sequence from TMV U1 RNA ($IRES_{MP228}^{U1}$) was also relatively high (Fig. 5B, column c).

Expression of the 3′-proximal GUS gene *in vivo*

To test whether the $IRES_{MP228}^{CR}$, $IRES_{MP228}^{U1}$, and $IRES_{CP146}^{CR}$ sequences also stimulate internal initiation of translation *in vivo*, two experimental approaches were used: (i) transient expression 35S promoter-based plasmids were constructed that specified the dicistronic mRNAs containing the first crTMV CP gene (or firefly luciferase, LUC, gene) and the GUS gene at the 3′-proximal position. The genes were separated by sequences mentioned above. Figure 6 shows that GUS gene expression was promoted *in vivo*, in tobacco protoplasts by dicistronic constructs even when the first gene was blocked by hairpin structure (Fig. 6C). In agreement with the results obtained in cell-free translation system (Fig. 5), the efficiencies of $IRES_{MP228}^{CR}$ and $IRES_{CP146}^{CR}$ (columns a and b in Figs. 6A–6C) were relatively similar, whereas the efficiency of an internal GUS gene translation directed *in vivo* by $IRES_{MP228}^{U1}$ was somewhat lower (column c in Figs. 6A–6C); (ii) microprojectile bombardment was applied for transient expression of the 3′-proximal GUS gene mediated by three IRES sequences listed above. The plasmids were introduced into *N. benthamiana* leaves by bombardment, and GUS activity was monitored by *in situ* staining. It has been concluded unambiguously that no blue foci representing GUS expression developed after bombardment with dicistronic constructs containing $U1_{CP146}^{SP}$ between the two coding regions (negative control). By contrast, a limited number (3–10 in a microscope vision area at magnification of ×20) of blue foci usually confined to individual cells developed invariably after bombardment

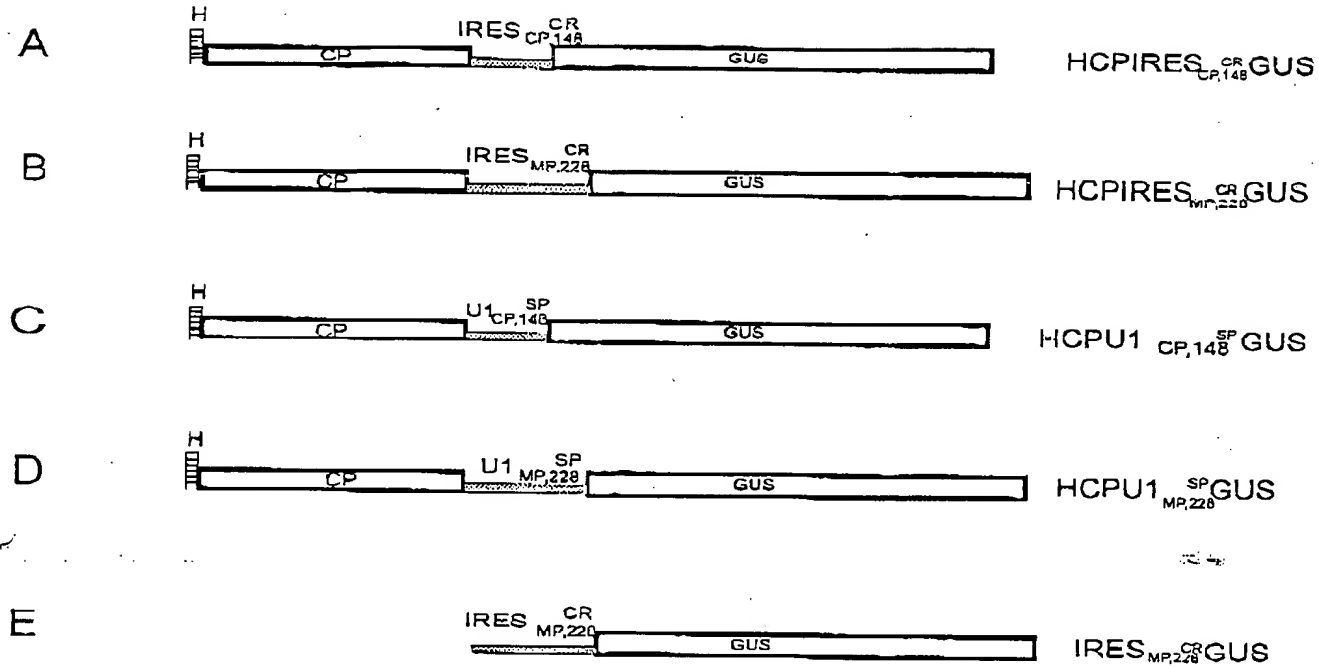

FIG. 3. Schematic representation of the dicistronic HCPGUS RNA transcripts. (A) HCPIRES$_{CP,148}^{CR}$GUS, the 5'-proximal CP gene with upstream sequence forming a potentially stable hairpin (H) and GUS gene are separated by the IRES$_{CP}^{CR}$ (see Ivanov *et al.*, 1997). (B) HCPIRES$_{MP,228}^{CR}$GUS, the 228-nt sequence located upstream of the MP gene of crTMV was inserted between the CP and GUS genes. (C) HCPU1$_{CP,148}^{SP}$GUS, the 148-nt region upstream of TMV U1 CP gene is inserted as the intercistronic spacer (see Ivanov *et al.*, 1997). (D) HCPU1$_{MP,228}^{SP}$GUS, the 228-nt sequence located upstream of the MP gene of TMV U1 was inserted as the intercistronic spacer. (E) IRES$_{MP,228}^{CR}$GUS, the GUS gene carrying the 228-nt IRES$_{MP,228}^{CR}$ sequence as the 5' leader.

with dicistronic constructs LUC-IRES$_{CP,148}^{CR}$GUS, LUC-IRES$_{MP,228}^{CR}$GUS and CP-IRES$_{CP,148}^{CR}$GUS. The same pattern of GUS staining was produced by the 35S-GUS plasmid pFF19G containing only the GUS gene (see Morozov *et al.*, 1997).

Some structural features of IRES$_{MP,228}^{CR}$

Definition of the structures which comprise the active IRES$_{MP}^{CR}$ in crTMV RNA is important toward understanding the mechanism of internal ribosome entry mediated by this element. Computer-assisted RNA secondary structure determinations suggest that the sequence, termed IRES$_{MP,228}^{CR}$, can be folded into a secondary structure, which could be divided tentatively into three regions marked by roman numerals in Fig. 7A. The region I (nts from 4649 to 4744 of crTMV RNA) apparently exists in the form of moderately stable stem-loop (−10.3 kcal/mol) structure. The region II of IRES$_{MP,228}^{CR}$ (nts 4745–4800) includes a potentially stable (−20.6 kcal/mol) hairpin structure and the region III (nts 4801–4875) contains a potentially unstable (−7.2 kcal/mol) structure upstream from AUG codon of the MP gene. In addition, the region III contains a 6-bp block (boxed in Fig. 7) that is conservative for crTMV and TMVU1 (see IRES$_{MP,75}^{U1}$ in Fig. 7B). Furthermore a homology of the nucleotide sequences upstream of crTMV and TMV U1 MP AUG codons is noteworthy (UUUGUUUGAUA-AUG and UUUGUUU-AUAG-AUG in crTMV and TMV U1, respectively). And finally, the conserved 6-bp block including the conserved UUUGUUU motif just upstream of the AUG codon could be revealed in TMV and crTMV IRES$_{MP,75}$ sequences. In crTMV the sequence is almost a direct repeat of three copies (Fig. 9).

The 75-nt regions upstream of the MP genes of crTMV and TMV U1 RNAs contain an internal ribosome entry site

To study the role of different parts of IRES$_{MP,228}^{CR}$ in internal initiation, two additional transcripts were constructed. The first was the dicistronic HCP(ΔI)IRES$_{MP,132}^{CR}$GUS transcript (Fig. 8A) with the 95-nt region I deleted from IRES$_{MP,228}^{CR}$. This truncated IRES$_{MP}$ retained the 3'-terminal 132-nt region used as an intercistronic spacer. The second dicistronic transcript HCP(ΔI-II)IRES$_{MP,75}^{CR}$GUS retained only the 3'-terminal 75-nt part of IRES$_{MP,228}^{CR}$ (Fig. 8A), whereas most of IRES$_{MP,228}^{CR}$ sequence was deleted. Figure 8B shows that deletion of regions I and II did not reduce the translation of the downstream GUS gene. To compare precisely the efficiency of internal initiation of translation promoted by the IRES$_{MP,228}^{CR}$ and IRES$_{MP,75}^{CR}$, the transcripts

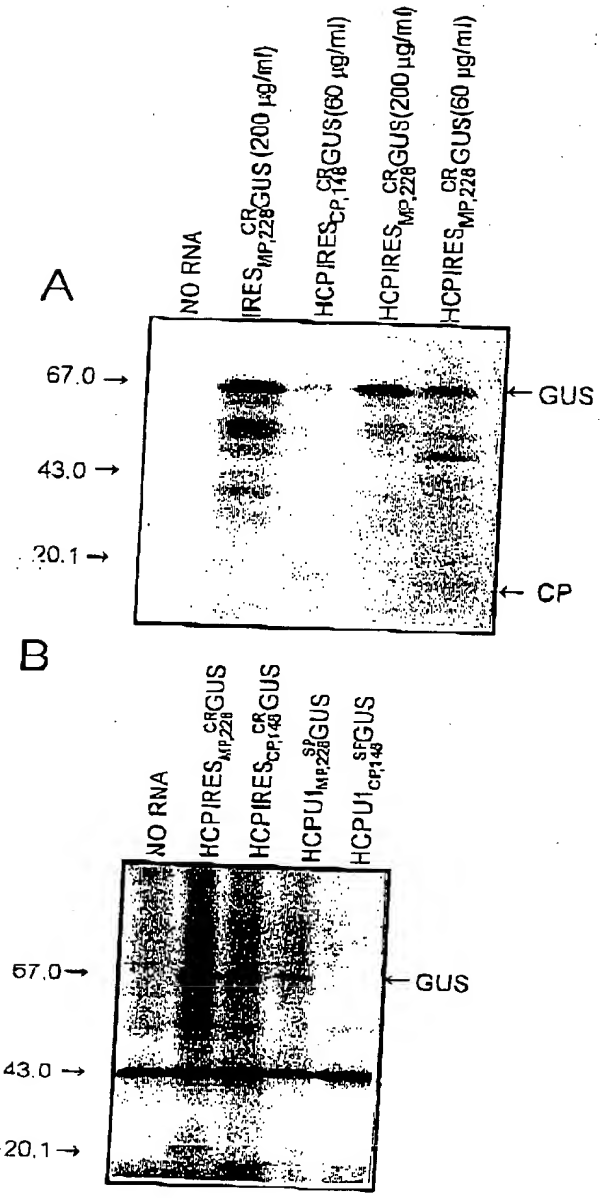

FIG. 4. Analysis of proteins directed in WGE (A) and RRL (B) by the dicistronic chimeric HCPGUS RNA transcripts with different crTMV and TMV U1 sequences inserted as the intercistronic spacers. The designations above the panels are described in Fig. 3. Concentration of transcripts is 40 μg/ml unless otherwise indicated.

(HCP-IRES$_{MP,228}^{CR}$GUS and HCP-IRES$_{MP,75}^{CR}$GUS) were translated in WGE and the activity of GUS produced *in vitro* was determined. Figure 9 shows that the reduction of the intercistronic sequence from 228 to 75 nt resulted in a significant increase of the ability of IRES$_{MP,75}^{CR}$ to promote the internal initiation of GUS gene translation (cf. columns a and d in Fig. 9). The equivalent 75-nt sequence from TMV U1 (IRES$_{MP,75}^{U1}$) also directed internal translation from dicistronic chimeric transcripts (Fig. 9c), however, the efficiency of translation mediated by IRES$_{MP,75}^{U1}$ was markedly lower than that mediated by IRES$_{MP,75}^{CR}$ (cf. columns c and d in Fig. 9).

Internal initiation of translation directed by the 5'-untranslated IRES$_{MP,75}^{CR}$ and IRES$_{MP,75}^{U1}$ sequences

In a series of experiments, two types of dicistronic transcripts that contained the MP, CP genes and the 3'UTR of crTMV or TMV U1 (Fig. 10A) were translated *in vitro*. The transcripts of the first type (IRES$_{MP,75}^{CR}$-MP-CP-3'UTR) represented the 3'-proximal 1610-nt fragment of crTMV RNA and contained the 75-nt 5'-nontranslated leader sequence derived from the replicase gene. The transcripts of the second type (IRES$_{MP,75}^{U1}$-MP-CP-3'UTR) contained the 75-nt 5'UTR derived from TMV U1 replicase gene and represented the 3'-proximal 1578-nt fragment of TMV U1 RNA (Fig. 10A). Therefore dicistronic transcripts of this series were analogous to tobamovirus I_2 sgRNAs. Figure 10B shows that translation of IRES$_{MP,75}^{CR}$-MP-CP-3'UTR (transcripts 1 in Fig. 10A) resulted in both MP and CP genes expression. The CP gene was expressed due to the presence of the IRES$_{CP,148}^{CR}$ between the MP and CP genes of crTMV RNA. The product of the CP gene translation was specifically immunoprecipitated by antibodies raised against crTMV CP (Ivanov *et al.*, 1997). The 5'-proximal MP gene was expressed from analogous transcript 5 (IRES$_{MP,75}^{U1}$-MP-CP-3'UTR) based on the TMV U1 RNA sequence. It has been demonstrated (Beachy and Zaitlin, 1977; Joshi *et al.*, 1983; Kiberstis *et al.*, 1983; Oliver *et al.*, 1986) that *in vitro* translation of TMV U1 I_2 sgRNA in WGE may result in the synthesis of two or more C-coterminal polypeptides with apparent molecular mass of 32 kDa and less that are initiated at an internal AUG codons in the same reading frame (nine internal AUG are indicated in Fig. 10A). On the other hand, the MP gene of crTMV contains only two AUG codons located at the middle region (Fig. 10A) and directs in WGE a polypeptide with electrophoretic mobility lower than that of TMV U1 MP (Fig. 10B). This is in agreement with the molecular mass of crTMV MP (~29 kDa) calculated from the deduced amino acid sequence of the MP (Dorokhov *et al.*, 1994). Taking into account the difference in methionine content between the MPs of crTMV and TMV U1, the MP-coding transcripts were translated in the presence of [^{14}C] amino acids mixture (Fig. 10B) to characterize the relative expression levels of the MP genes. To confirm unambiguously that translation of the MP genes of crTMV and TMV U1 proceeds by internal initiation on the 5'-terminal IRES-containing nontranslated leaders, we constructed dicistronic transcripts that contained a stable hairpin (H) structure immediately at the 5' end and upstream of the nontranslated 75-nt leaders (HIRES$_{MP,75}^{CR}$-MP-CP-3'UTR

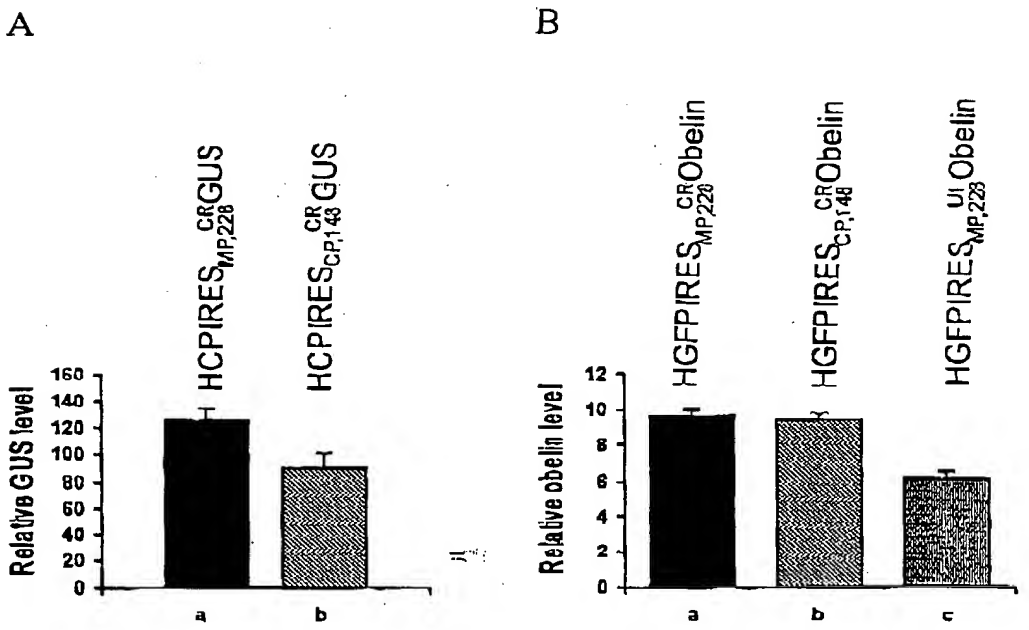

FIG. 5. Relative efficiencies of IRES$_{MP,228}^{CR}$ and IRES$_{CP,148}^{CR}$ in directing internal initiation of GUS gene (A) and obelin gene (B) *in vitro* translation. The dicistronic RNA transcripts (HCP-spacer-GUS; HGFP-spacer-obelin) translated in WGE contained different sequences as an intercistronic spacers: IRES$_{MP,228}^{CR}$ (a), IRES$_{CP,148}^{CR}$ (b), and IRES$_{MP,228}^{U1}$ (c). The GUS or obelin activity in translation samples was expressed in terms of a relative expression levels as described under Materials and Methods. The mean values for 5–6 (GUS in A) and 12 (obelin in B) individual translation samples are given. Standard error bars are presented.

and HIRES$_{MP,75}^{U1}$-MP-CP-3'UTR in Fig. 10A). Figure 10B shows that these transcripts (2 and 4) produced the 30- and 32-kDa proteins, respectively. By contrast, no translation of the MP gene could be detected from a control dicistronic transcript 3 (HPL$_{80}$-MP-CP-3'UTR) that contained downstream of H structure a polylinker-derived nonphysiological 80-nt sequence instead of IRES$_{MP,75}^{CR}$. Expression of the CP from this transcript (Fig.10B) was apparently mediated by IRES$_{CP,148}^{CR}$. It should be noted that translation of the 5'-proximal MP gene was completely abolished when the dicistronic analogue of crTMV sgRNA I_2 contained the hairpin structure directly at the 5' terminus but did not contain the 75-nt IRES$_{MP}$ leader upstream of the MP gene (see the construct TBS MP CP in Figs. 2 and 3, Ivanov *et al.*, 1997). These data taken together suggested that the MP genes of crTMV and TMV U1 could be expressed from dicistronic analogues of I_2 sgRNAs containing the 75-nt leader sequences between the hairpin and MP gene (transcripts 2 and 4 in Fig. 10A) by a mechanism that was 5' end independent. This strongly implies that expression of the MP genes from uncapped I_2 sgRNAs of at least some of tobamoviruses might proceed by internal ribosome entry mechanism mediated by IRES$_{MP}$ element located in the 5'-leader sequence. This conclusion does not exclude that a traditional ribosome-scanning mechanism of the I_2 sgRNA MP gene expression operates concurrently. The capacity of IRES$_{MP,75}$ to promote the MP production from the 5'-terminal H-structure-carrying transcripts 2 and 4 was markedly lower than from H-lacking transcripts (1 and 5 in Fig. 10B). Figure 10B also shows that the translational efficiency of IRES$_{MP,75}^{CR}$ was somewhat higher than that of IRES$_{MP,75}^{U1}$.

In a separate experiment, we tested if the 5'-terminal H-structure-carrying HIRES$_{MP,75}^{CR}$ sequence also can mediate an internal initiation of translation *in vivo*. Figure 10C shows that the 3'-proximal GUS gene expression can be promoted by IRES$_{MP,75}^{CR}$ in tobacco mesophyll protoplasts electroporated with the 35S promoter-based HIRES$_{MP,75}^{CR}$-GUS cDNA constructs.

An alternative explanation of our results would be that the sequence thought to be an IRES$_{MP}$ is instead an efficient cleavage site, particularly susceptible to nucleases in translation extracts. To show that the downstream cistrons were not being translated from degraded RNAs, the transcripts were incubated in translation system (WGE) and RNA integrity was determined by Northern blot analysis. We found that no significant changes in electrophoretic mobility or integrity were observed after incubation in WGE of dicistronic IRES$_{MP,228}^{CR}$- and IRES$_{MP,228}^{U1}$-containing transcripts (data not shown). In similar experiments, the stability of dicistronic constructs HCPIRES$_{CP,148}^{CR}$GUS and HCPU1$_{CP,148}^{SP}$GUS in translation system has been demonstrated by Ivanov *et al.* (1997).

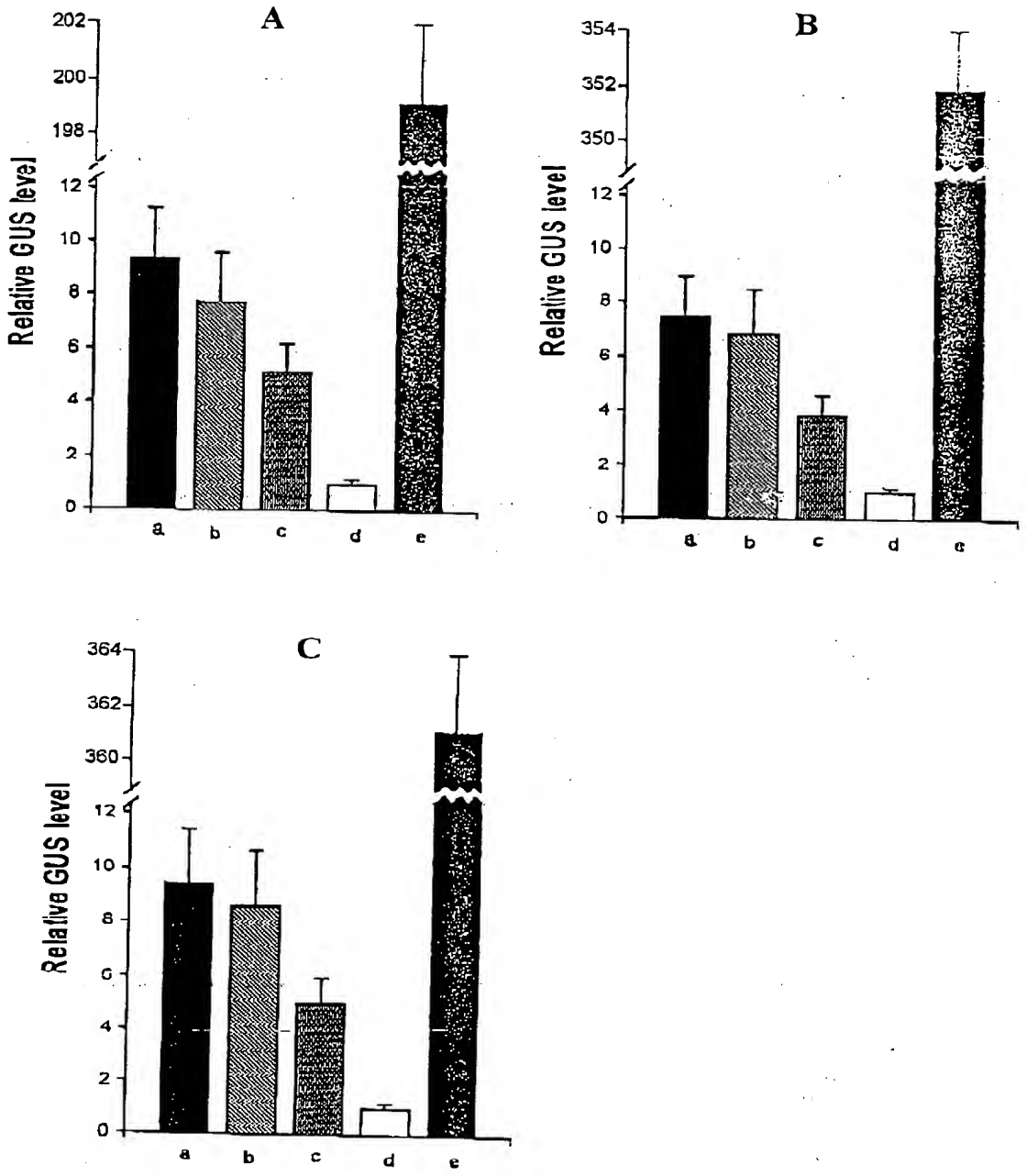

FIG. 6. Transient expression of the 3'-proximal GUS gene of dicistronic constructs in tobacco protoplasts. Three types of the 35S promoter-based dicistronic constructs CP-spacer-GUS (A), LUC-spacer-GUS (B), and H-LUC-spacer-GUS (C) used for protoplasts transfection contained different sequences as an intercistronic spacers: $IRES_{MP339}^{CR}$ (a), $IRES_{CP148}^{CR}$ (b), $IRES_{MP256}^{U1}$ (c), and $U1_{CP148}^{SP}$ (d). Monocistronic construct pFF19G with GUS gene (e). The GUS activity was expressed as described under Materials and Methods. The nonspecific background GUS activity associated with nontransfected protoplasts (0.35 relative units) was subtracted throughout. The mean values for five (A) and three (B, C) independent experiments are given.

DISCUSSION

According to the ribosome scanning model (Kozak, 1989, 1992), the internal ORF_s of the polycistronic eukaryotic mRNA would not be accessible to ribosomes. However, an alternative mode of a cap-independent translation initiation has been described for a variety of viral and

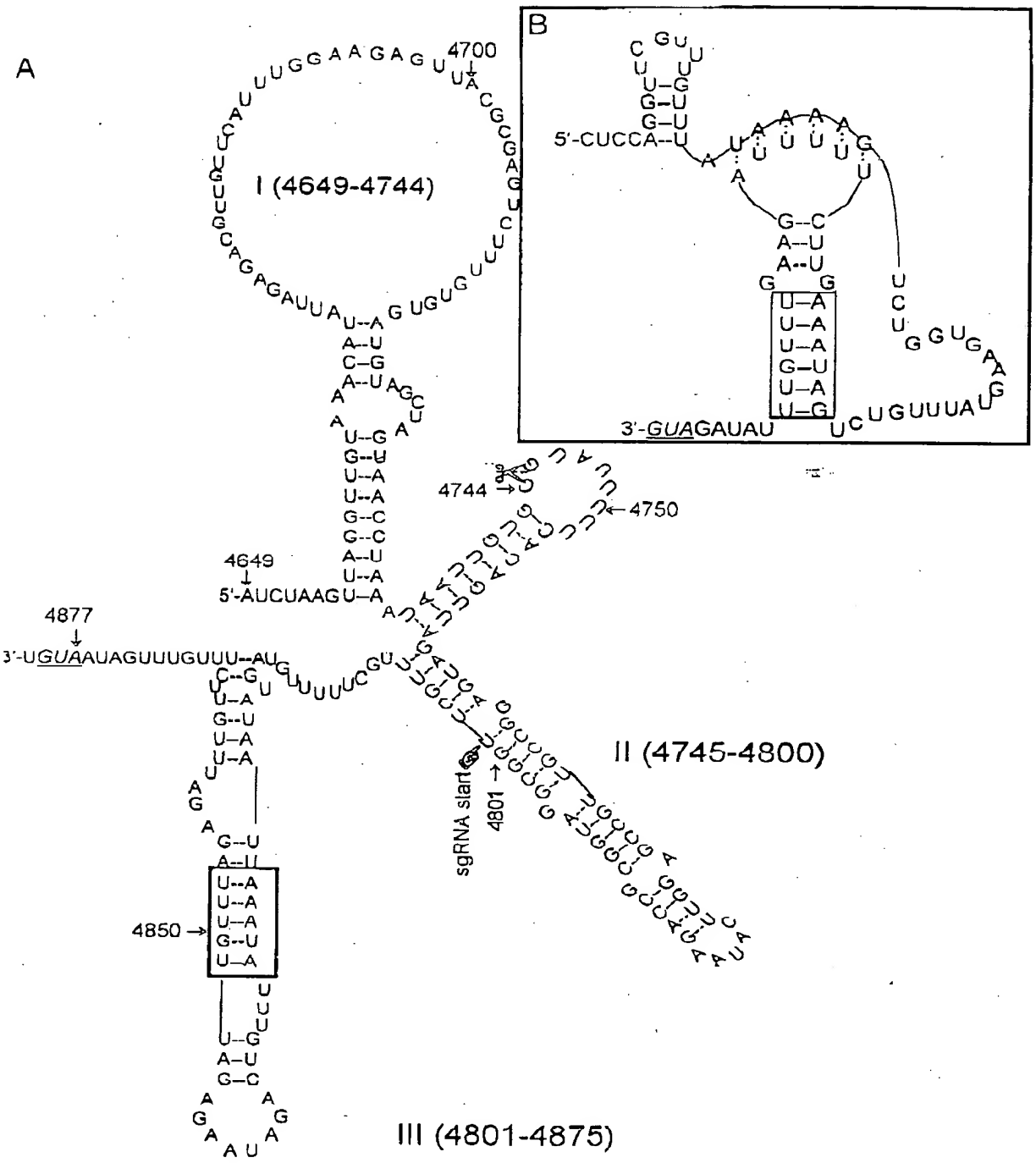

FIG. 7. Computer-generated secondary structure of the $IRES_{MP,726}^{CM}$ (A) and $IRES_{MP,72}^{U}$ (B) regions upstream from the MP gene AUG codons (underlined). Roman numerals denote the regions of $IRES_{MP,729}^{CA}$ (see text); scissors point to the positions resulting in deletion mutant $(\Delta I)IRES_{MP,732}^{CA}$. The 5'-terminal nucleotide of sgRNA I_2 is indicated (sgRNA-start). The deletion mutant $IRES_{MP,72}^{U}$ (B) is described in the text.

cellular mRNAs (reviewed by Belsham and Sonenberg, 1996; Pain, 1996).

It long has been known that only the 5′-proximal gene of tobamovirus genomic RNA can be directly translated by ribosomes. A dicistronic uncapped sgRNA called I_2 directs translation of only MP, whereas a second, capped

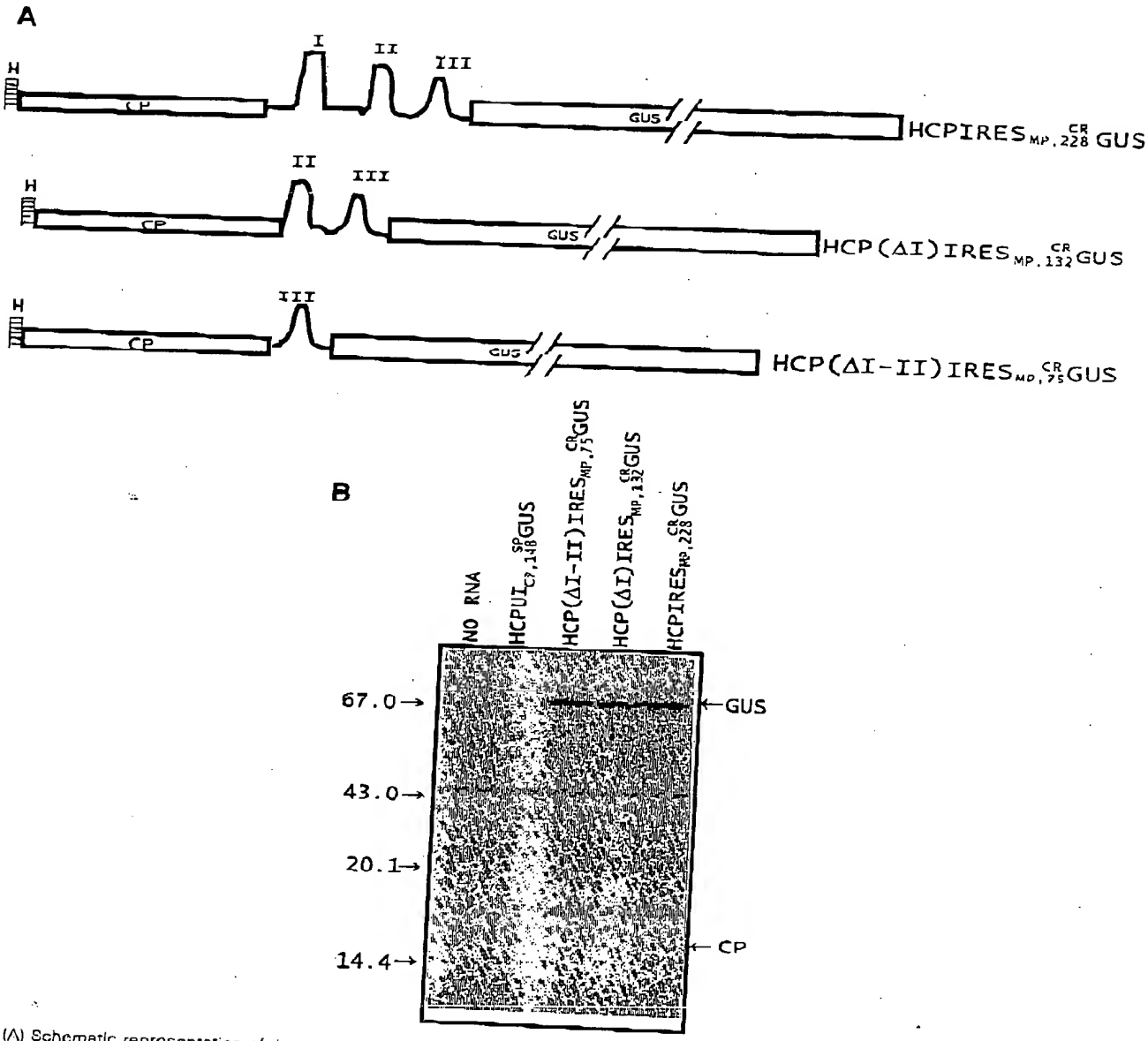

FIG. 8. (A) Schematic representation of the dicistronic chimeric HCPIRES$_{MP,228}^{CR}$GUS transcript and its deletion mutants. Roman numerals denote the regions of IRES$_{MP,228}^{CR}$ described in the text and Fig. 7A. (B) Analysis of proteins directed in RRL by dicistronic transcripts HCPGUS containing the 5′-truncated IRES$_{MP}^{CR}$ sequences (see A). Concentration of transcripts is 40 μg/ml.

monocistronic sgRNA directs synthesis of the CP (reviewed by Palukaitis and Zaitlin, 1986). Recently we have isolated and sequenced the genome of a new tobamovirus infecting the members of Cruciferae family (crTMV) (Dorokhov *et al.*, 1993, 1994). In the previous paper (Ivanov *et al.*, 1997), we have reported that unlike the RNA of typical tobamovirus TMV U1, internal translation of the 3′-proximal CP gene of crTMV can be mediated *in vitro* by a specific 148-nt sequence element (IRES$_{CP,148}^{CR}$) upstream of the CP gene.

In this study, we show that the 228-nt region upstream of the MP gene of crTMV RNA contains an internal ribosome entry site that allows 5′-end-independent internal initiation of translation on synthetic dicistronic transcripts including CP-IRES$_{MP,228}^{CR}$-MP (Figs. 1 and 2), CP-IRES$_{MP,228}^{CR}$GUS (Figs. 4 and 5A), and GFP- IRES$_{MP,228}^{CR}$-obelin (Fig. 5B). The MP, GUS, and obelin genes are expressed from these dicistronic transcripts even when the translation of the first gene is abolished by hairpin structures inserted immediately at the 5′ termini. These results indicate that the IRES$_{MP,228}^{CR}$ mediates internal *in vitro* translation of

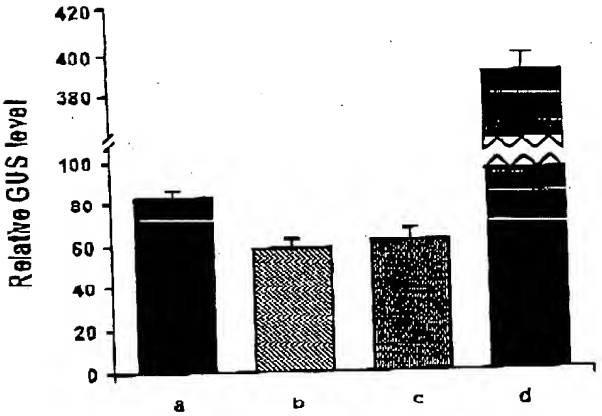

FIG. 9. The 75-nt regions upstream of the MP genes of crTMV and TMV U1 RNA contain IRES. The dicistronic RNA transcripts HCP-spacer-GUS translated in WGE contained the following sequences as an intercistronic spacer: $IRES_{MP,228}^{CR}$ (a), $IRES_{CP,148}^{CR}$ (b), $IRES_{MP,75}^{U1}$ (c), and $IRES_{MP,75}^{CR}$ (d). The GUS activity was expressed as described under Materials and Methods. The mean values for 5–10-individual translation samples are given.

different reporter genes despite their 3'-proximal localization.

Interestingly, the crTMV $IRES_{CP,148}^{CR}$ and $IRES_{MP,228}^{CR}$ are active either in animal-cells-derived (RRL) or plant-cells-derived (WGE) systems. By contrast, poliovirus and encephalomyocarditis virus (EMCV) RNAs are nonfunctional in WGE, whereas EMCV RNA is highly functional in RRL (Dorner *et al.*, 1984; Jang *et al.*, 1988).

Then we compared the relative efficiencies of $IRES_{MP,228}^{CR}$ and $IRES_{CP,148}^{CR}$ in directing internal translation by inserting each IRES into a single dicistronic construct. Determination of GUS (Fig. 5A) and obelin (Fig. 5B) activity produced by the 3'-proximal reporter gene showed that the efficiencies of two IRESs did not differ dramatically although the activity of the $IRES_{MP,228}^{CR}$ was somewhat higher. To gain insight in the functional peculiarity of an $IRES_{MP,228}^{CR}$, the equivalent 228-nt sequence from TMV U1 RNA was taken as the intercistronic sequence ($U1_{MP,228}^{SP}$) contemplated as the negative control. Contrary to expectations, the $U1_{MP,228}^{SP}$ was capable of mediating internal translation from dicistronic transcripts (Figs. 4B and 5B). By analogy with the $IRES_{MP,228}^{CR}$ of crTMV, this sequence was referred to as $IRES_{MP,228}^{U1}$. Although the functional significance of these observations was obscure, our results indicated that both tobamoviruses contained an IRES elements upstream of their MP genes that allowed 5'-end-independent *in vitro* translation of the second cistron when placed into the intercistronic region of chimeric dicistronic RNA. It is apparent that the conformational properties of $IRES_{MP}$ sequences may be totally different in the synthetic dicistronic transcripts and genomic *Tobamovirus* RNAs. Nonfunctionality of the $IRES_{MP,228}$ sequences within the genomic TMV U1 and crTMV RNAs could be due to the extensive secondary structure and inaccessibility of this region to ribosomes in full-length tobamovirus RNA.

It was not excluded that requirements for internal initiation of translation in a plant cell may differ from requirements in a cell-free translation system. It has been reported that the region upstream of the CP gene of tobacco necrosis virus genomic RNA promoted internal translation in WGE; however, this region was functionally inactive *in vivo*, in tobacco protoplasts (Meulewaeter *et al.*, 1992). Two lines of evidence indicate that the $IRES_{MP,228}^{CR}$, $IRES_{MP,228}^{U1}$, and $IRES_{CP,148}^{CR}$ promote the downstream gene expression *in vivo*. The 3'-proximal GUS gene was expressed from dicistronic IRES-carrying 35S promoter-based constructs in tobacco mesophyll protoplasts (Fig. 6) and after microprojectile bombardment of *N. benthamiana* leaves as visualized by *in situ* GUS staining. The relative levels of GUS expression in protoplasts transfected by monocistronic GUS gene-carrying construct pFF19G (Fig. 6a) were dramatically higher than expression of the GUS gene from bicistronic IRES-containing constructs (Fig. 6, a–c). It should be noted that these cDNA constructs varied in size from ~1.8 kb (pFF196G: Fig. 6e) to 4 kb (HLUC-IRES-GUS; Fig. 6C), i.e., the molar amount of the capped monocistronic GUS mRNA transcribed *in vivo* from pFF19G should markedly exceed the productivity of bicistronic constructs. Although the results presented in Fig. 6e cannot be interpreted directly in terms of a quantitative comparison of GUS gene expression from the respective monocistronic and bicistronic transcripts, it can be suggested that translational expression of monocistronic GUS mRNAs is higher than that mediated by the $IRES_{MP,228}^{CR}$ (Fig. 6a).

To study the role of different parts of $IRES_{MP,228}^{CR}$ in internal initiation, two deletion mutants were constructed that retained the 3'-terminal 132-nt [(ΔI) $IRES_{MP,132}^{CR}$] and 75-nt [(ΔI-II) $IRES_{MP,75}^{CR}$] regions of $IRES_{MP,228}^{CR}$, respectively (Fig. 8A). It was found that deletion of the 3'-terminal regions I and II (see Fig. 7) did not reduce (Fig. 8B) and even increased (Fig. 9) the ability of $IRES_{MP,75}^{CR}$ to direct internal translation of GUS gene from dicistronic transcripts, suggesting that these sequences were not involved in $IRES_{MP,228}^{CR}$ function. Although the boundaries of $IRES_{MP}^{CR}$ have not been defined precisely, deletion analysis allowed to conclude that the IRES element is contained within the 75-nt region upstream of the MP gene (region III in Fig. 7). The functional role of the separate sequence elements of $IRES_{MP,75}$ essential for the MP gene expression is obscure. Our results indicated that $IRES_{MP,75}^{CR}$ that is unusually short, is markedly distinct from IRESs of picornaviruses and other eukaryotic mRNAs described so far. Furthermore this observation has drawn attention to the fact that the length of the 75-nt $IRES_{MP,75}^{CR}$ was very close to that of the 5'-untranslated leader sequences of I_2 sgRNAs of tobamoviruses:

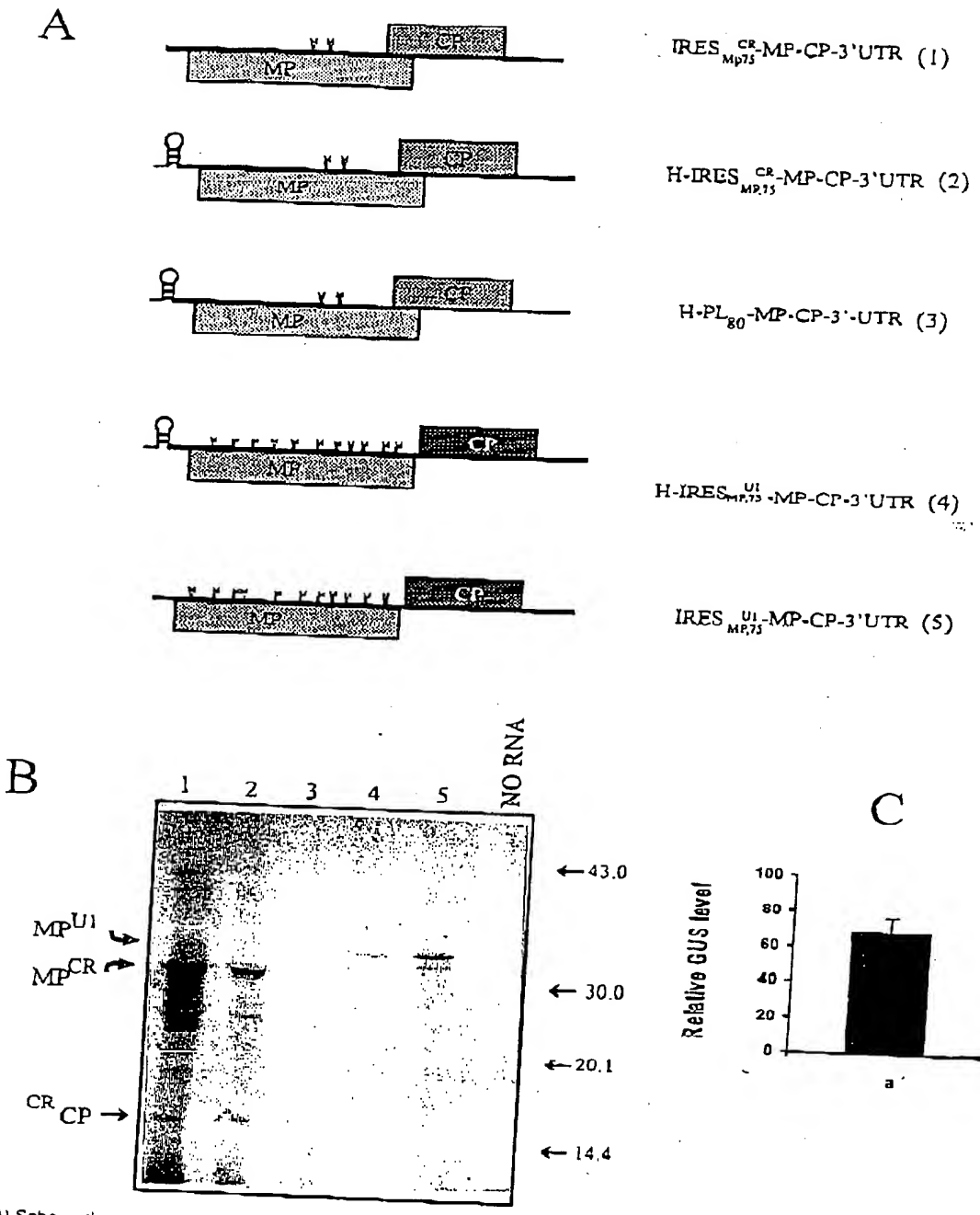

FIG. 10. (A) Schematic representation of the dicistronic analogues of I_2 sgRNAs of crTMV and TMV U1 and their derivatives with the 5′-terminal hairpin (H) structures. The transcripts 1, 2, 4, and 5 contained the 75-nt nontranslated leader sequences derived from the regions of crTMV or TMV U1 RNAs upstream from their MP genes ($IRES_{MP,75}^{CR}$ and $IRES_{MP,75}^{U1}$, respectively). The transcripts 2–4 contained the 5′-terminal hairpin structure (H) upstream of the 75-nt 5′UTR. The transcript 3 (HPL_{80}-MP-CP-3′UTR) taken as a negative control contained a polylinker-derived nontranslated 80-nt sequence located upstream of the MP gene. The positions of internal methionine (AUG) codons in the MP genes are indicated (M). The MP and CP genes of TMV U1 and crTMV are distinguished by different shading. (B) Analysis of proteins directed in WGE by the dicistronic analogues of I_2 sgRNAs of crTMV and TMV U1 in the presence of [^{14}C] amino acids mixture. Arabic numerals above the panel correspond to transcripts 1–5 presented in (A). The positions of crTMV and TMV U1 MPs and of crTMV CP are indicated. (C) Transient expression of the 3′-proximal GUS gene in tobacco protoplasts electroporated with 35S promoter-based H-$IRES_{MP,75}^{CR}$GUS (a) and H-$U1_{CP,148}^{SP}$GUS (b) constructs. Relative GUS activity was expressed as described under Materials and Methods. The nonspecific background GUS activity associated with nontransfected protoplasts (0.82 relative units) was subtracted throughout. The mean values for three independent samples are given.

the 5'UTR of TMV U1 I_2 RNA consists of 75 nts (Lehto *et al.*, 1990) and the 5'UTR of crTMV I_2 RNA is 75 nt long (V. A. Efimov *et al.*, personal communication).

The IRES-mediated translation is typical for mRNAs that contain their long and highly structured 5'UTR_s. These RNAs retain IRES elements so that ribosomes may bypass stable secondary structures at their 5' termini. It is hard to predict whether the 5'-leader sequences of I_2 sgRNAs can actually hinder the MP gene translation; however, another feature namely the lack of a 5' m^7G-cap in TMV U1 I_2 RNA (and presumably, in crTMV I_2 RNA) should make the I_2 RNA a poor template for translation. Consequently the question arises as to whether the 5'-proximal MP gene of subgenomic RNA I_2 is translated by a "scanning ribosome" mechanism or whether their 5'-terminal untranslated $IRES_{MP,75}^{CR}$ and $IRES_{MP,75}^{U1}$ sequences are capable of mediating internal ribosome binding on the 5'UTR of RNA I_2. To test experimentally this hypothesis, the dicistronic uncapped T7 RNA transcripts 5'UTR-MP-CP-3'-UTR were synthesized (Fig. 10A) that were structurally equivalent to dicistronic I_2 sgRNAs produced *in vivo* by TMV U1 and crTMV, respectively. It was found that the 5'-proximal MP genes could be translated from RNAs of this type even when they contained a stable hairpin structure immediately at the 5' end, upstream of the nontranslated 75-nt sequences ($HIRES_{MP,75}^{U1}$–MP-CP-3'UTR and $HIRES_{MP,75}^{CR}$-MP-CP-3'UTR in Fig. 10B). The presence of H structure upstream of the 5'UTR ruled out the possibility of the MP gene expression by a ribosome scanning mechanism; however, the MP genes still were expressed. The results indicated that the efficiency of $IRES_{MP,75}^{CR}$ was somewhat higher in mediating the MP production as compared with $IRES_{MP,75}^{U1}$ (cf. lanes 2 and 4 in Fig 10B). This is in agreement with the results showing that the relative efficiency of $IRES_{MP,75}^{CR}$ in directing internal initiation of GUS gene translation was significantly higher than that of $IRES_{MP,75}^{U1}$ (cf. columns c and d in Fig. 9). It is possible that the $IRES_{MP,75}^{CR}$ sequence is functionally active when located at the 5' end of I_2 sgRNA (but not in the full-length genomic context) because of competing RNA folding with TMV sequences not present in the reporter gene or I_2 RNA context. This is consistent with the fact that the $IRES_{MP,75}^{CR}$ is more active than H- $IRES_{MP,228}^{CR}$ (Fig. 9).

The 30-kDa MP is produced transiently, early in the infection (Watanabe *et al.*, 1984; Moser *et al.*, 1988; Lehto *et al.*, 1990) and in low amounts (Moser *et al.*, 1988). Little is known concerning the regulation of the 30-kDa MP gene expression (for review, see Dawson and Lehto, 1990). Our results suggest that initiation of *in vitro* translation of the MP gene can occur by direct binding of ribosomes to the 5'-untranslated leader of I_2 sgRNA. Moreover the results presented in Fig. 10C indicate that the 5'-terminal H-structure-carrying $HIRES_{MP,75}^{CR}$ sequence also can mediate an internal translation of GUS gene *in vivo*, in tobacco mesophyll protoplasts. Evidently our conclusion that translation of the MP gene can be promoted by an $IRES_{MP}$ element does not exclude that a traditional ribosome scanning mechanism of this gene expression operates concurrently.

MATERIALS AND METHODS

Viruses and RNA

TMV U1 and crTMV were isolated from systemically infected *Nicotiana tabacum* L. cv. Samsun plants as described previously (Dorokhov *et al.*, 1994).

Plasmid constructs

The plasmids pCP, pH-CP, pH-CP-$IRES_{CP,148}^{CR}$GUS, and pH-CP-$U1_{CP,148}^{SP}$GUS were described previously (Ivanov *et al.*, 1997).

The series of CP-MP transcripts (Fig. 1). The construct pH-CP contained T7 promoter, the 102-nt inverted tandem repeat (hairpin structure H in Fig. 1G and CP gene of crTMV inserted into pBluescript SKII+ plasmid as a PCR product). The MP gene of crTMV containing the 5' terminal (i) 52-nt polylinker-derived leader or (ii) the 228-nt sequence upstream of the MP gene (LMPCP in Fig. 2A, Ivanov *et al.*, 1997) was digested with *Xba*I–*Sac*I and cloned into pH-CP using *Xba*I–*Sac*I sites to obtain final constructs pH-CP-MP and pH-CP-$IRES_{MP,228}^{CR}$-MP, respectively.

The series of dicistronic H-CP-GUS transcripts (Fig. 3). To obtain pH-CPIRES$_{MP,228}^{CR}$GUS the 228-nt *Hin*dIII–*Nco*I fragment from pH-CP-$IRES_{MP,228}^{CR}$-MP was cloned together with *Nco*I–*Xba*I digested GUS fragment from pH-CP-$IRES_{CP,148}^{CR}$GUS into pH-CP (Fig. 1B) using *Hin*dIII and *Xba*I sites. In this series of constructs, the H-structure was truncated to 68 nts using *Cla*I site.

The PCR product was obtained by RT–PCR of TMV U1 genomic RNA with (i) direct primer (a*gaattc*CCTAAAGTTGATCTCGAAACT) corresponding to the 228-nt region upstream the MP gene and containing *Eco*RI site and (ii) reverse primer (gat*cccatgg*ATAAACAAACTTCTAAAAAGA) with *Nco*I site. The PCR product was digested with *Eco*RI–*Nco*I and cloned together with *Nco*I–*Xba*I-digested GUS fragment into pH-CP (Fig. 1B) using *Eco*RI and *Xba*I sites to create pH-CP-$U1_{MP,228}^{SP}$GUS (Fig. 3D).

The series of transcripts with truncated $IRES_{MP,228}^{CR}$ and $IRES_{MP,228}^{U1}$. The PCR product was obtained by RT–PCR of crTMV genomic RNA with (i) direct primer (a*gaattc*GTATTTTCACAGTTAGATGAG) corresponding to the 132-nt region upstream the MP gene and reverse primer mentioned above. The PCR product was digested with *Hin*dIII and *Nco*I and cloned together with *Nco*I–*Xba*I-digested GUS fragment into pH-CP using *Hin*dIII and *Xba*I sites to obtain pH-CP-(ΔI)IRES$_{MP,132}^{CR}$GUS (Fig. 8A). To construct pH-CP-(ΔI-II)$IRES_{MP,75}^{CR}$GUS, a product was obtained by RT–PCR of crTMV genomic RNA with direct primer (a*gaattc*GTTTGCTTTTTGTAGTAT) ($DP_{MP,75}^{CR}$) cor-

responding to the 75-nt region upstream the MP gene (Fig. 8A).

pH-CP-U1$_{MP,228}$^{SP}GUS was used to truncate U1^{SP} using RT–PCR of crTMV genomic RNA by similar procedure with (*i*) direct primer (*agaattc*CCTCCAGGTTCGTTTGTTTAT) (DP$_{MP,75}$^{U1}) corresponding to the 75-nt region upstream the MP gene and (*ii*) reverse primer (*gatcccatg*gATAAACAAACTTCTAAAAAGA). PCR product was cloned between *Eco*RI and *Nco*I sites, as described above resulting in pH-CP-IRES$_{MP,75}$^{U1}GUS.

The series of dicistronic H-GFP-obelin transripts. *Eco*RI–*Kpn*I polylinker fragment from pBluescript SKII+ was cloned together with *Kpn*I–*Bam*HI fragment from GFP plasmid into pBluescript SKII+ using *Eco*RI and *Bam*HI. Resulting plasmid pH-GFP was digested with *Hin*dIII, filled with Klenow fragment, and ligated back to eliminate *Eco*RI and *Hin*dIII sites. Then it was restricted with *Nco*I, filled with Klenow fragment and ligated back to eliminate *Nco*I at the very beginning of GFP gene.

To obtain pH-GFP-IRES$_{CP,148}$^{CR}-obelin, the obelin gene was constructed as a PCR product using plasmid pOL6 (Illarionov *et al.*, 1995) as a template, *Nco*I-site-containing direct and *Xba*I-containing reverse primers. The 148-nt *Eco*RV–*Nco*I fragment, containing IRES$_{cr}$ of crTMV from pH-CP-IRES$_{CP,148}$^{CR}GUS, was cloned together with *Nco*I-modified obelin gene, digested with *Nco*I and *Xba*I into pH-GFP using filled *Bam*HI site and *Xba*I site.

The 228-nt *Eco*RI–*Nco*I fragment from pH-CP-IRES$_{MP,228}$^{CR}GUS was cloned into pH-GFP-IRES$_{CP,148}$^{CR}-obelin using *Eco*RI and *Nco*I sites to obtain pH-GFP-IRES$_{MP,228}$^{CR}-obelin.

PCR product (see description of the pH-CP-IRES$_{MP,228}$^{U1}GUS construct) was digested with *Nco*I and cloned into pH-GFP-IRES$_{CP,148}$^{CR}-obelin using *Eco*RV (blunt) and *Nco*I sites to obtain pH-GFP-IRES$_{MP,228}$^{U1}-obelin.

The series of crTMV and TMV U1 I$_2$-like transcripts (Fig 1A). pHSK. Polylinker fragment *Hin*dIII–*Kpn*I (filled) from pBluescript SKII+ was cloned into pBluescript SKII+ using *Hin*dIII and *Sma*II sites. Resulting plasmid was digested with *Cla*I and ligated back to eliminate *Hin*dIII site. Required restriction sites were introduced into obtained plasmid by cloning into *Bam*HI–*Xba*I sites the *Bam*HI–*Xba*I polylinker fragment from pGEM7Z. Obtained vector contained a 34-nt inverted repeat that produces a stable hairpin structure in corresponding transcript (see Fig. 7Ah in Ivanov *et al.*, 1997).

pIRES$_{MP,75}$^{CR}-MP-CP-3′UTR. PCR product obtained from crTMV cDNA clone (Dorokhov *et al.*, 1994) using DP$_{MP,75}$^{CR} and reverse primer complementary to 3′-terminal part of crTMV MP gene was digested with *Hin*dIII and *Eco*RI and cloned together with *Eco*RI–*Sac*I fragment from crTMV cDNA clone LMP-CP (Ivanov *et al.*, 1997) into pBluescript SKII+ vector using *Hin*dIII and *Sac*I sites.

pH-IRES$_{MP,75}$^{CR}-MP-CP-3′UTR. *Cla*I (filled)-*Sac*I fragment from pIRES$_{MP,75}$^{CR}-MP-CP-3′UTR was cloned into pHSK using *Bam*HI (filled) and *Sac*I sites.

pH-PL$_{80}$-MP^{CR}-CP^{CR}-3′UTR. *Bam*HI–*Sac*I fragment from pTBS-MP-CP-3′UTR (Ivanov *et al.*, 1997) was cloned into pHSK using *Bam*HI and *Sac*I sites. Resulting plasmid contained the 80-nt polylinker-derived spacer between H and initiation codon of the MP gene.

pIRES$_{MP,75}$^{U1}-MP-CP-3′UTR. PCR product obtained from TMV U1 cDNA clone using DP$_{MP,75}$^{U1} and reverse primer complementary to TMV U1 MP gene was digested with *Eco*RI and *Hin*dIII and cloned together with *Hin*dIII–*Kpn*I fragment from TMV U1 cDNA clone TMV204 (Lehto *et al.*, 1990) into pGEM3Z using *Eco*RI and *Kpn*I sites.

pH-IRES$_{MP,75}$^{U1}-MP-CP-3′UTR. *Eco*RI–*Xba*I fragment from pIRES$_{MP,75}$^{U1}-MP-CP-3′UTR was cloned into pHSK using *Eco*RI and *Xba*I sites.

The 35S promoter-based GUS-expression constructs. To construct the 35S promoter-based clones dicistronic constructs, CP-spacer-GUS, LUC-spacer-GUS, H-LUC-spacer-GUS and H-IRES$_{MP,75}$^{CR}GUS were cloned in the pFF19 plasmid (Timmermanns *et al.*, 1990).

In vitro transcription and translation

The plasmids of the series pCP-MP, pH-IRES$_{MP,75}$^{CR}-MP-CP-3′UTR, pIRES$_{MP,75}$^{CR}-MP-CP-3′UTR, and pH-PL$_{80}$-MP^{CR}-CP^{CR}-3′UTR were linearized by Ec1136II (Fermentas, Lithuania). The plasmids of the series pH-CP-GUS, pH-GFP-obelin, pIRES$_{MP,75}$^{U1}-MP-CP-3′UTR, and pH-IRES$_{MP,75}$^{U1}-MP-CP-3′UTR were linearized by *Xba*I. The linearized plasmids were transcribed *in vitro* as described earlier (Tomashevskaya *et al.*, 1993), and agarose gel electrophoresis of RNA transcripts confirmed that they were intact. The RNA concentration was quantified by spectrophotometry.

Synthetic uncapped mRNA transcripts were translated in RRL as described earlier (Ivanov *et al.*, 1997) or in WGE according to the manufacturer's (Promega and Boehringer Mannheim) protocol in the presence of [^{35}S]methionine or [^{14}C] amino acids mixture for 60 min at 25°C. Radiolabeled translation products were analysed by SDS–PAGE and localized by autoradiography on the dried gel. It should be noted that the 42-kDa band revealed in RRL corresponds to a product of endogenous RNA translation.

Stability of dicistronic transcripts upon *in vitro* translation

The dicistronic IRESs containing transcripts obtained by standard procedure were translated in WGE, and their integrity was examined after translation by Northern blot analysis. Ten microliters of translation sample was treated with phenol, then with phenol/chloroform (1:1), and finally with chloroform. Total RNA from the samples was precipitated with ethanol and dissolved in water. The sample was treated according to standard Northern blot hybridization (Sambrook *et al.*, 1989) with *in vitro*-transcribed ^{32}P-labeled specific riboprobes.

Protoplasts preparation and transfection: determination of GUS and obelin activity

The following procedures were used: (i) the protoplasts were isolated from *N. tabacum* (cv. W38) leaves as described by Saalbach *et al.* (1996). Aliquots of 4 × 10^5 protoplasts were co-electroporated (electric impulse of 1 ms at 750 V/cm) with 10 μg of pFF19-based dicistronic DNA constructs "CP-spacer-GUS" and 10 μg of pCLN DNA containing the firefly luciferase (LUC) gene (Callis *et al.*, 1987) and incubated for 18 h at 25°C in the dark. GUS activity was measured as relative light units (RLU) by TROPIX GUS-light kit following the manufacturer's protocol and using a LKB 1251 Wallac luminometer. LUC activity was measured using Promega kit according to the manufacturer's protocol. LUC activity was measured to have an internal control. GUS activity was calculated by normalizing it with LUC activity in each sample; (ii) the protoplasts were isolated from *N. tabacum* (cv. SR-1) leaves and transfected with 15 μg of pFF19-based dicistronic constructs (LUC-spacer-GUS and H-LUC-spacer-GUS) using PEG-mediated DNA transfection (Negrutiu *et al.*, 1987). GUS activity was determined according to Jefferson (1987). For each experiment, background GUS activity associated with nontransfected protoplasts was subtracted throughout. Protein concentration was estimated using a Bio-Rad protein assay kit based on the method by Bradford (1976).

To characterize the relative efficiencies of different IRESs in directing internal translation in transfected protoplasts, GUS activity was presented in terms of a relative expression levels. Extremely inefficient but detectable expression level was directed by dicistronic constructs containing $U1_{CP,148}^{SP}$ as an intercistronic spacer. In each experiment, this level was taken as one unit of GUS activity. The same way was used for measuring relative expression levels of GUS and obelin genes in WGE. Obelin activity was measured according to Matveev *et al.* (1995). For each experiment, background GUS (or obelin) activity associated with WGE without exogenous RNA was subtracted throughout.

Particle bombardment

Particle bombardment was performed using the flying disc method with high-pressure helium-based apparatus as described by Morozov *et al.* (1997).

Secondary structure folding

The secondary structure folding of the $IRES_{MP,228}^{CR}$ and $IRES_{MP,75}^{U1}$ was performed using the GENEBEE package.

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